FEBS Letters 585 (2011) 2682–2687

journal homepage: www.FEBSLetters.org

Ago2/miRISC-mediated inhibition of CBP80/20-dependent translation and thereby abrogation of nonsense-mediated mRNA decay require the cap-associating activity of Ago2

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ARTICLE INFO

Article history:
Received 28 May 2011
Revised 19 July 2011
Accepted 28 July 2011
Available online 10 August 2011

Edited by Tamas Dalmay

Keywords:
Ago2
MicroRNA
NMD
CBP80/20
eIF4E

ABSTRACT

Nuclear cap-binding protein (CBP) 80/20-dependent translation (CT) is one of the targets for miRNA-mediated gene silencing. Here, we provide evidence that human argonaute 2 (Ago2) competes with CBP80/20 for cap-association, inhibiting CT and thus nonsense-mediated mRNA decay (NMD), which is tightly coupled to CT. Tethering of Ago2, but not of Ago2F2V2 which lacks cap-association activity, to the 3′UTR of PTC-containing mRNA abrogates NMD. Immunoprecipitation using CBP80 antibody reveals that Ago2, but not Ago2F2V2, inhibits the binding of CBP80/20 to cap structure. Our observations provide molecular insight into the cross-talk between miRNA-mediated gene silencing, CT, and NMD.

Structured summary of protein interactions:
AGO2 physically interacts with **GW182** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

Eukaryotic mRNA translation is mediated by two distinct cap-binding proteins: (i) the nuclear cap-binding protein (CBP) complex (CBC), a heterodimer of CBP80 and CBP20, and (ii) the cytoplasmic cap-binding protein, eukaryotic translation initiation factor 4E (eIF4E), each of which has the ability to recruit ribosome(s) to initiate translation [1,2]. The cap structure at the 5′-end of premature mRNAs (pre-mRNAs) is recognized by CBP80/20 in the nucleus. Mature messenger ribonucleoproteins (mRNPs) harboring CBP80/20 are exported from the nucleus to the cytoplasm via the nuclear pore complex (NPC) [1,3,4]. During mRNP export, CBP80/20 recruits a ribosome to the 5′-end of the mRNA, probably via its interaction with CBP80/20-dependent translation initiation factor (CTIF), which is localized to the cytoplasmic side of the nuclear envelope and interacts with eIF3 [5]. This round of translation is called the "first (or pioneer) round of translation" or, to specify the cap-binding protein, "CBP80/20-dependent translation (CT)" [1,5]. It should be noted that CT is tightly coupled to the mRNA surveillance mechanism, nonsense-mediated mRNA decay (NMD), by which premature termination codon (PTC)-containing mRNAs are selectively recognized and eliminated before expression of truncated proteins [3,4]. After CT, CBP80/20 is replaced by eIF4E. A series of protein interactions of eIF4E-eIF4GI/II-eIF3 trigger efficient recruitment of 40S, directing multiple rounds of translation called "steady-state translation" or "eIF4E-dependent translation (ET)" [2].

MicroRNAs (miRNAs) are small noncoding RNAs that post-transcriptionally regulate gene expression of target mRNAs by base-pairing with 3′-untranslated region (3′UTR) [6,7]. Mature miRNAs are complexed with Argonaute family proteins, forming the so-called "miRNA-induced silencing complex (miRISC)". MiRISC is loaded onto the 3′UTR of target mRNAs and functions to inhibit translation of target mRNA in various ways [6,7].

Previously, our group found that human Ago2 is loaded onto CBP80/20-bound mRNAs and that artificial tethering or loading of Ago2/miRISC onto 3′UTR of PTC-containing mRNAs inhibits CT efficiency and thus NMD [8]. However, the exact steps in the inhibition of CBP80/20-dependent translation mediated by Ago2/miRISC need to be elucidated in future studies. In this study, we show that Ago2/miRISC-mediated NMD inhibition is dependent on the ability of Ago2 to associate with the cap structure. Our observations provide molecular insight into novel cross-talk between miRNA-mediated gene silencing, CT, and NMD.

2. Materials and methods

2.1. Cell culture, plasmid transfection, semi-quantitative RT-PCR and quantitative real-time PCR

The details are provided in the Supplementary materials.

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doi:10.1016/j.febslet.2011.07.047

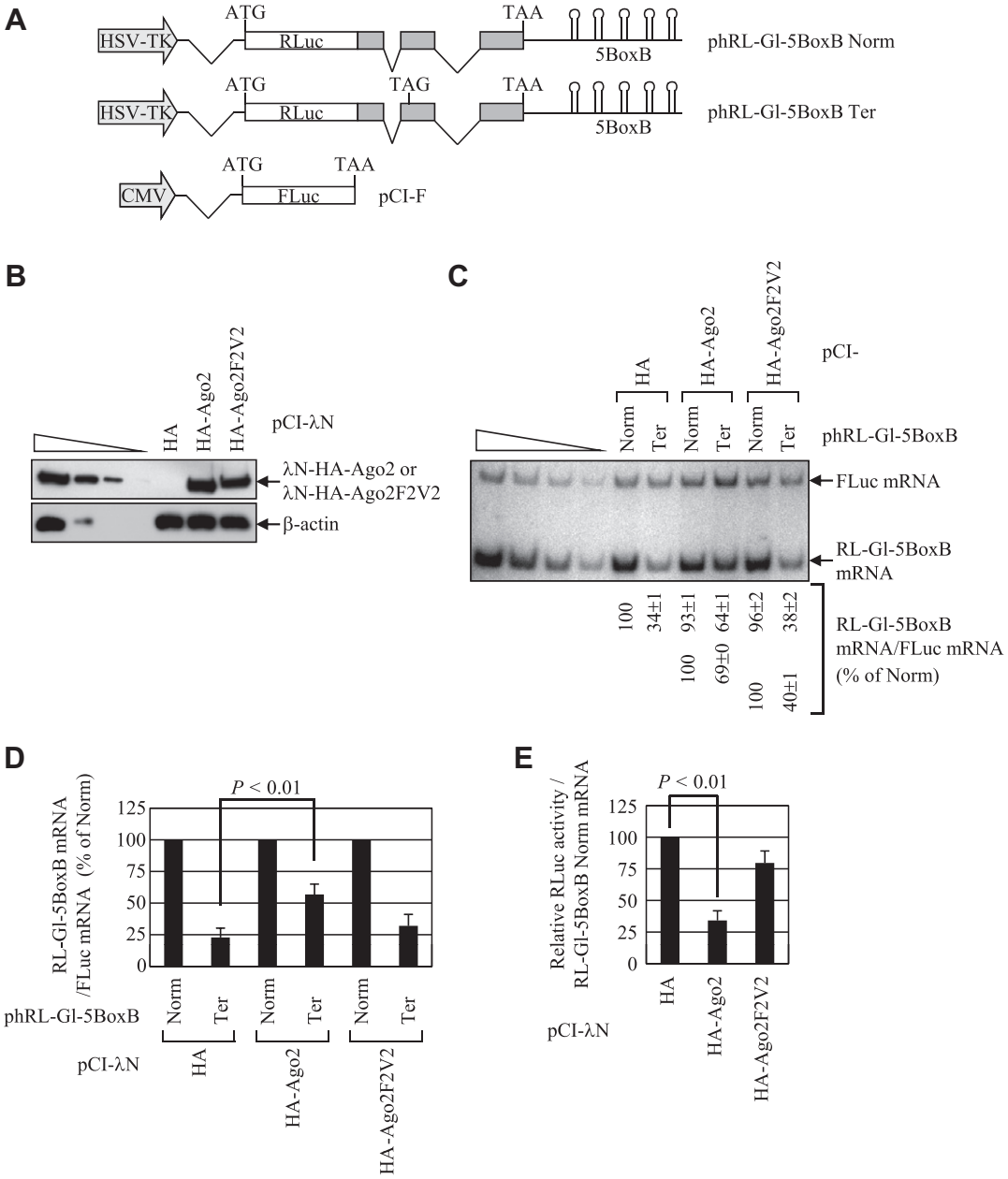

Fig. 1. Artificial tethering of human Ago2, but not Ago2F2V2, at the 3′UTR abrogates NMD. (A) Schematic representations of the tethering NMD reporter constructs. Tethering NMD reporter plasmid phRL-Gl-5BoxB Norm or Ter contains, in sequence: *Renilla* luciferase (RLuc) cDNA without translation termination codon (open box), β-globin (Gl) gene (the grey boxes) either Norm or 39Ter (which harbors the PTC at the 39th residue), a normal translation termination codon, and five tandem repeats of 19-nucleotide binding site (5boxB) of the λ bateriophage antiterminator protein N (λN). (B–E) HeLa cells were transiently co-transfected with 1 μg of the indicated effector plasmid, 0.1 μg of tethering NMD reporter construct, and 0.1 μg of pCI-F. (B) Western blotting of λN-HA-Ago2, λN-HA-Ago2F2V2. (C) Semi-quantitative (sq) RT-PCR of RL-Gl-5BoxB mRNAs and FLuc mRNAs. The levels of RL-Gl-5BoxB mRNAs were normalized to the levels of FLuc mRNA. The normalized level of RL-Gl-5BoxB Norm mRNA in the presence of λN-HA was set to 100% (upper numbers). Alternatively, normalized levels of RL-Gl-5BoxB Norm mRNA in the presence of each effector were set to 100% (lower numbers). (D) Real-time RT-PCR of RL-Gl-5BoxB mRNAs and FLuc mRNAs. As in (C), except that total RNAs were analyzed by real-time PCR. (D) Translational efficiency of RL-Gl-5BoxB mRNAs. The relative RLuc activity (RLuc activity/FLuc activity) was normalized to the relative amount of RL-Gl-5BoxB mRNA (RL-Gl-5BoxB Norm/FLuc mRNA). Normalized translation efficiency of RL-Gl-5BoxB Norm mRNA in the presence of λN-HA was set to 100%.

2.2. Immunoprecipitation and Western Blotting

Immunoprecipitation (IP) was performed as described previously [5,8,9]. Cell extracts or immunopurified proteins were electrophoresed in SDS-polyacrylamide (6–12%) and transferred to HyBond ECL nitrocellulose (Amersham). The following antibodies were used: HA (Roche), eIF4E (BD biosciences), CBP80 [8], GW182 (a gift from Dr. Marvin J. Fritzler), and β-actin (Sigma).

2.3. Dual luciferase assay

Dual luciferase assays were performed according to the manufacturer's protocol (Promega) and detected with a Glomax 20/20 Luminometer (Promega).

2.4. Cap-association assay using m^7GTP-sepharose

The details are provided in the Supplementary materials.

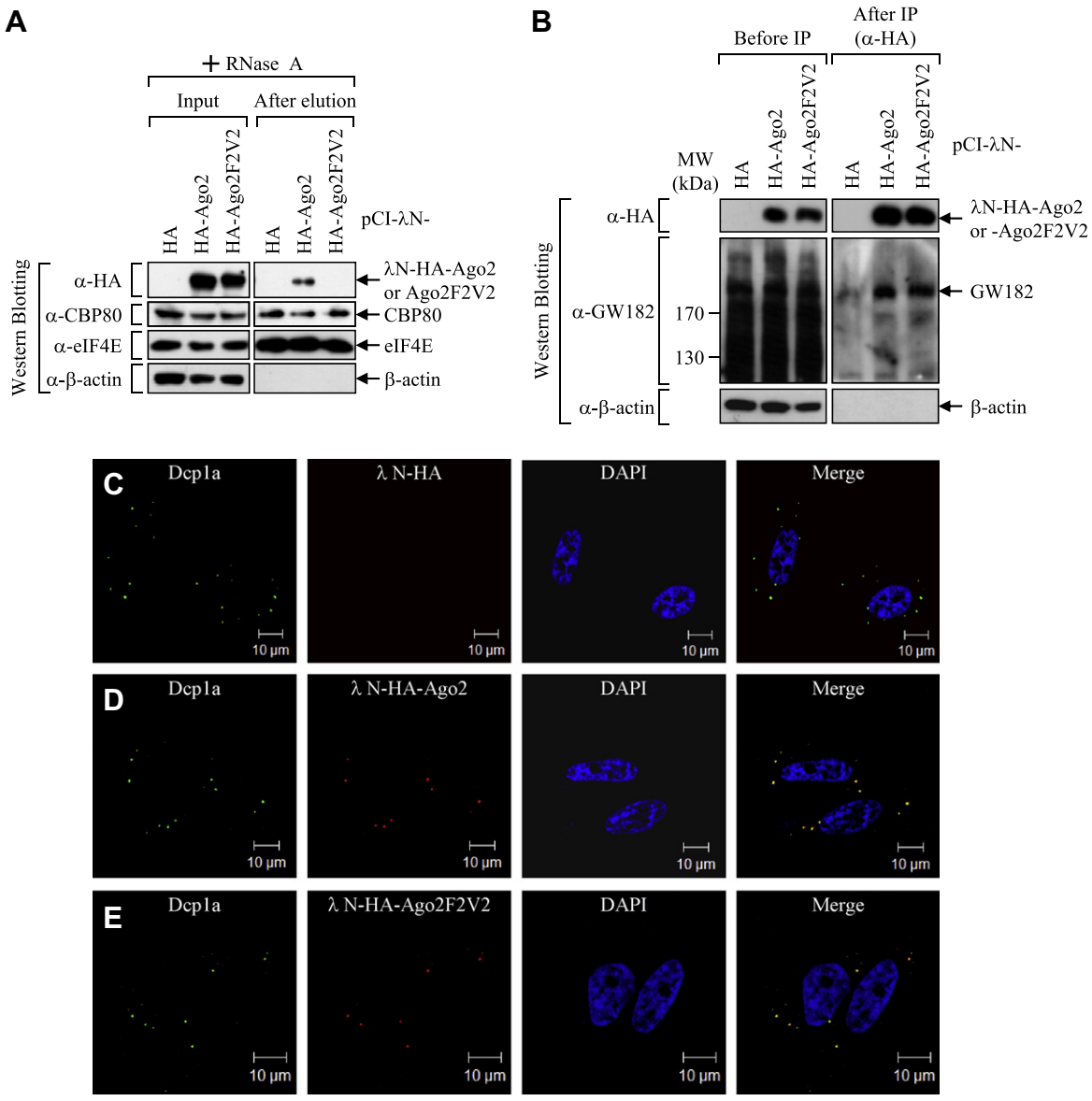

Fig. 2. F2V2 substitutions affect the cap-associating activity of Ago2 without significantly affecting its interaction with GW182 and P-bodies localization. (A) Cap-association assay of λN-HA-Ago2 and λN-HA-Ago2F2V2. The total-cell extracts (Input) and the cap-bound protein samples were analyzed by Western blotting using the indicated antibodies. (B) Western blotting of endogenous GW182 in IPs of λN-HA-Ago2 and λN-HA-Ago2F2V2. (C–E) HeLa cells were transiently transfected with 2 μg of plasmid expressing λN-HA (C), λN-HA-Ago2 (D), or λN-HA-Ago2F2V2 (E) and then stained with α-HA antibody and α-Dcp1a antibody. Dcp1a served as the positive control for P-bodies localization.

3. Results

3.1. NMD is abrogated by tethering of Ago2, but not Ago2F2V2, to the 3′UTR of PTC-containing mRNA

Previously, our group found that artificial tethering of Ago2 to 3′UTR of the NMD reporter RL-Gl-5BoxB Norm or Ter mRNAs with the λN/boxB system [10] inhibits the NMD of RL-Gl-5BoxB Ter mRNA [8]. Using the same system, we tested which step is targeted in Ago2/miRNA-mediated NMD inhibition. To this end, we employed the Ago2F2V2 mutant, in which two phenylalanines in the MC domain are substituted to valines and which lacks cap -associating activity [10]. HeLa cells were transiently co-transfected with three plasmids: (i) an effector plasmid expressing λN-HA, λN-HA-Ago2, or λN-HA-Ago2F2V2, (ii) a tethering NMD reporter plasmid expressing either RL-Gl-5BoxB Norm or Ter mRNA, and (iii) a reference plasmid expressing firefly luciferase (FLuc) mRNA (Fig. 1A). Two days after transfection, total-cell RNAs and proteins were harvested and analyzed by Western blotting (Fig. 1B), semi-quantitative RT-PCR (sqRT-PCR) using specific oligonucleotides and α-[^{32}P]-dATP (Fig. 1C), and quantitative real-time PCR (Fig. 1D).

The results revealed that, even if comparable amounts of λN-HA-Ago2 and λN-HA-Ago2F2V2 were expressed (Fig. 1B), tethering of λN-HA-Ago2, but not of λN-HA-Ago2F2V2, abrogated NMD of RL-Gl-5BoxB mRNA by 2-fold (Fig. 1C). The sqRT-PCRs in Fig. 1C were further confirmed by quantitative real-time PCR (Fig. 1D). The quantitative real-time PCR results were very similar to those obtained by sqRT-PCR, demonstrating that our methodology is sufficiently quantitative to detect the differences. Consistent with a previous report [10], tethering of λN-HA-Ago2 repressed overall translation of RL-Gl-5BoxB Norm mRNA to 34% compared to that obtained from tethering of λN-HA (Fig. 1E), without significantly changing the mRNA level (Fig. 1C), suggesting that translational repression by tethered Ago2 was efficient under these conditions. On the other hand, tethering of λN-HA-Ago2F2V2 marginally affected the overall translation of RL-Gl-5BoxB Norm mRNA

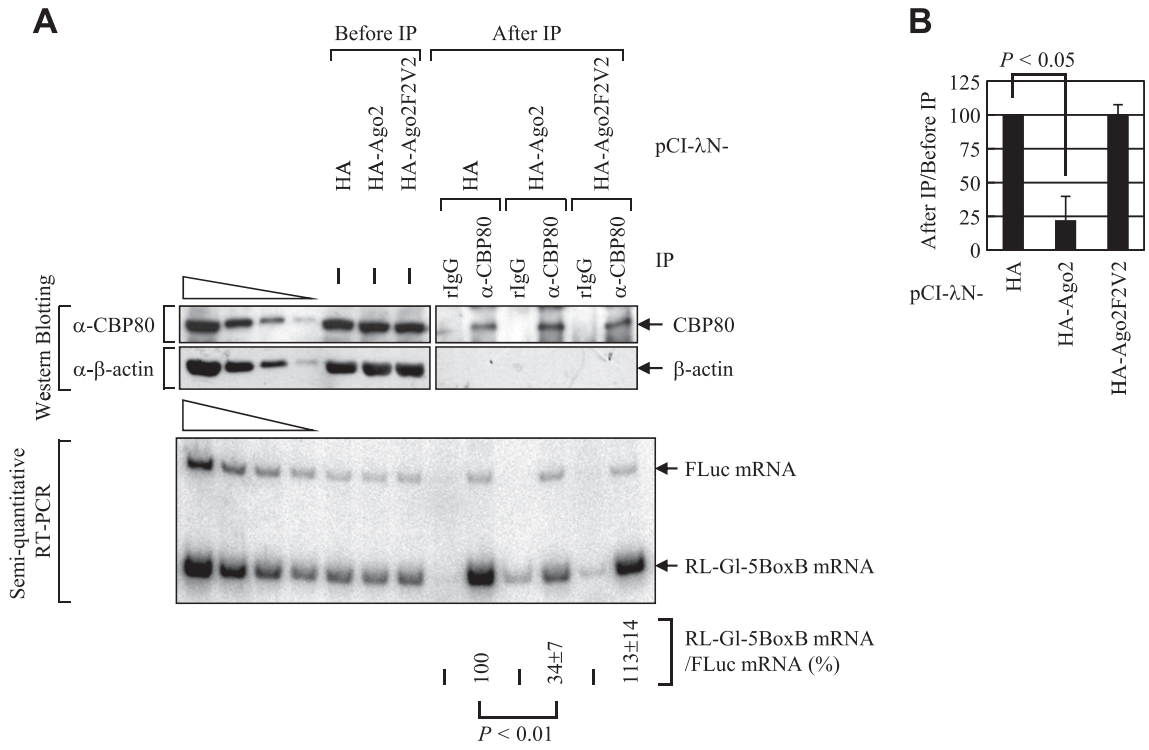

Fig. 3. Competition between Ago2 loaded onto 3′UTR and CBP80/20 for cap-association. Cos-7 cells were transiently co-transfected with (i) plasmid expressing λN-HA, λN-HA-Ago2, or λN-HA-Ago2F2V2, (ii) phRL-Gl-5BoxB Norm, and (iii) reference plasmid pCI-F. Total-cell proteins and RNAs before or after IP with α-CBP80 antibody were analyzed by Western blotting (A, upper), sqRT-PCR (A, lower), and quantitative real-time PCR (B), respectively. Each level of RL-Gl-5BoxB mRNA was normalized to the level of FLuc mRNA. The normalized co-immunopurified RL-Gl-5BoxB mRNA level was then normalized to the level of the normalized RL-Gl-5BoxB mRNA before IP. The normalized level of co-immunopurified RL-Gl-5BoxB mRNA obtained in IP of λN-HA was set to 100%.

(Fig. 1E). All of these results suggest that Ago2/miRISC-mediated CT and NMD inhibition involves the cap-associating activity of Ago2.

3.2. Introduction of F2V2 substitutions abolishes cap-associating activity of Ago2 without affecting its binding to GW182 and its localization to processing bodies

We examined the molecular features of Ago2 and Ago2F2V2, because the cap-associating activity of Ago2 and subsequent gene silencing remain controversial [10–15]. First, transiently expressed λN-HA-Ago2 wild-type, endogenous CBP80, and eIF4E, but not λN-HA-Ago2F2V2, were significantly associated with m^7GTP-Sepharose resin (Fig. 2A) even after RNase A treatment, suggesting that (i) Ago2 has extrinsic or intrinsic cap-associating activity and (ii) F2V2 substitutions affect the cap-associating activity of Ago2. Second, comparable amounts of endogenous GW182 were detected in the IP of λN-HA-Ago2 and λN-HA-Ago2F2V2 (Fig. 2B), suggesting that (i) F2V2 substitutions do not significantly affect the interaction between Ago2 and GW182 and (ii) Ago2F2V2 is inactive in silencing CT and NMD due to the lack of cap-associating activity, but not due to the loss of binding to GW182 under these conditions. Third, the localization of Ago2 into processing bodies (P-bodies), where translationally silenced mRNAs or mRNAs that are destined to be degraded are localized, was not affected by F2V2 substitutions (Fig. 2C–E). All these results suggest that the introduction of F2V2 substitutions into Ago2 abolishes the cap-associating activity of Ago2 without affecting other molecular features.

3.3. Ago2 inhibits the binding of CBP80/20 to the cap structure

The above observations led us to hypothesize that Ago2/miRISC loaded onto 3′UTR may compete with CBP80/20 for cap-association, because Ago2 has the ability to associate with the cap structure (Fig. 2). This hypothesis was clearly demonstrated by analyzing the abundance of mRNAs co-purified with endogenous CBP80 under conditions in which Ago2 or Ago2F2V2 was tethered at the 3′UTR of the mRNA. If tethered Ago2 at the 3′UTR competed with CBP80/20 for cap-association, less co-immunopurified mRNAs would be detected in the IP of CBP80. The results showed that, although comparable amounts of λN-HA-Ago2 and λN-HA-Ago2F2V2 were expressed and although comparable levels of endogenous CBP80 were immunopurified (Fig. 3A, upper), tethering of λN-HA-Ago2, but not λN-HA-Ago2F2V2, inhibited the co-immunopurification of RL-Gl-5BoxB mRNA by 3-fold (Fig. 3A, lower). The sqRT-PCRs in Fig. 3A were further confirmed by quantitative real-time PCR (Fig. 3B). All of these results indicate that Ago2/miRISC loaded onto 3′UTR associates with the cap structure directly or indirectly and thus competes with CBP80/20 for cap-association.

4. Discussion

Here we provide evidence that Ago2/miRISC associates with the cap structure, competing out CBP80/20 from the cap structure and consequently inhibiting CT and NMD. Based on our results, we propose possible models in which Ago2/miRISC regulates the CT efficiency and NMD (Fig. 4). First, if an mRNA had no miRNA-binding sites and no PTC, it would undergo both CT and ET (Fig. 4A). Second, if an mRNA had miRNA-binding sites but no PTC, its expression would be silenced at both CT and ET (Fig. 4B). Third, if an mRNA had no miRNA-binding sites but contained a PTC, it would be targeted for NMD and hence downregulated in abundance (Fig. 4C). Finally, if an mRNA had both miRNA-binding sites and a PTC, the CT of the mRNA would be silenced through the competition for cap-association between Ago2/miRISC loaded onto the 3′UTR and CBP80/20. NMD would consequently be silenced. Even if CBP80/20 is replaced by eIF4E, the ET of the mRNA would still be silenced through the

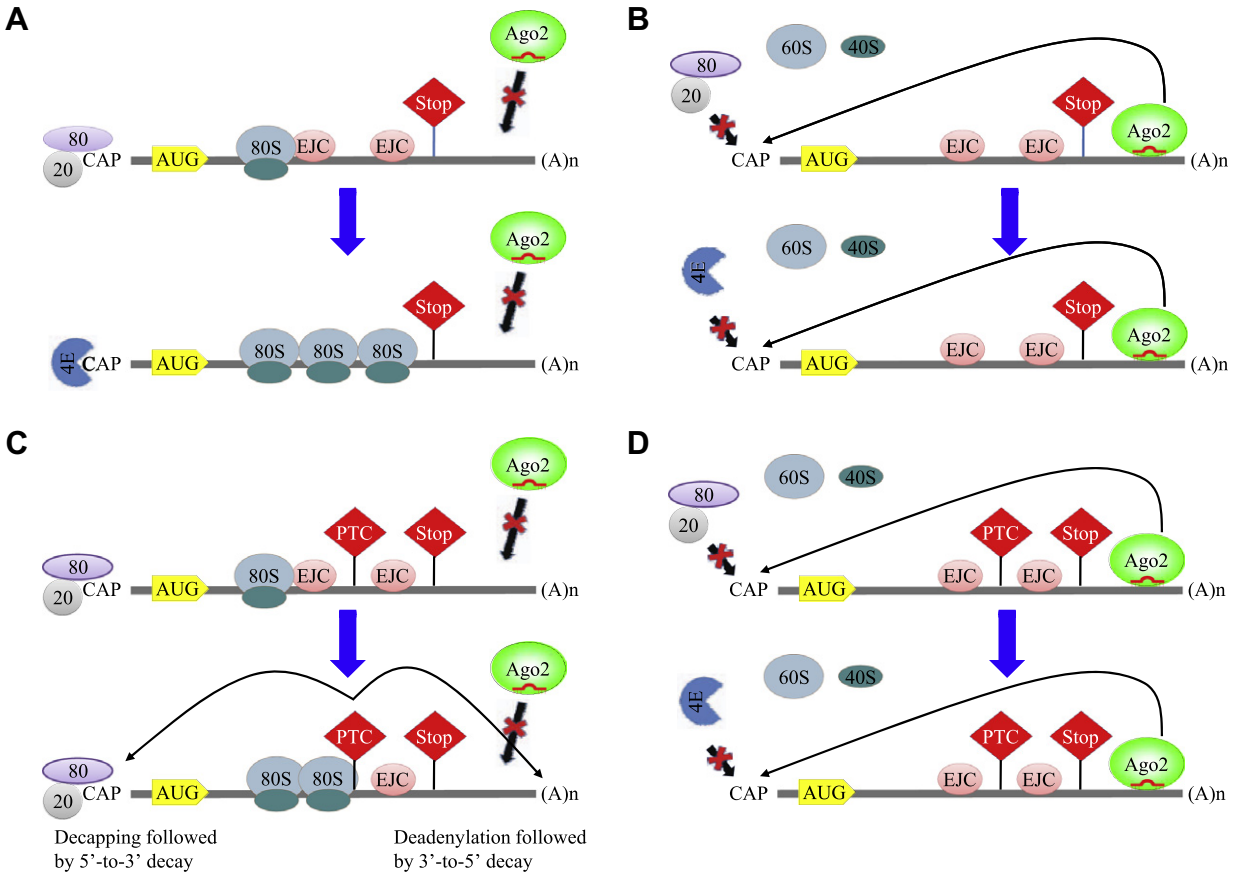

Fig. 4. Models illustrating cross-talk between Ago2/miRISC-mediated gene silencing, CT, ET, and NMD in mammalian cells. The details are described in the discussion.

competition between Ago2/miRISC and eIF4E for cap-association (Fig. 4D).

In our study, we found that, although Ago2F2V2 fails to associate with cap structure (Fig. 2A and 3) and to inhibit translation of RL-Gl-5BoxB Norm mRNA (Fig. 1E), Ago2F2V2 associates with GW182 and is localized to P-bodies (Fig. 2E), similar to Ago2. These results suggest that the localization of Ago2 into P-bodies is irrelevant for Ago2-mediated translational silencing. This is reminiscent of mRNA decay in P-bodies. The enrichment of many mRNA-degrading enzymes in P-bodies may help mRNAs to be degraded more quickly. However, NMD and microRNA-mediated mRNA decay are not affected by the removal of microscopically visible P-bodies using siRNA against factors critical for the formation of P-bodies [16–18], suggesting that the formation of microscopically visible P-bodies is not sufficient for efficient mRNA decay and is a consequence of mRNA degradation. Similarly, it is possible that the formation of microscopically visible P-bodies is dispensable for Ago2-mediated translational silencing.

Considering previous reports and our study, it seems that the molecular behaviors of mammalian Ago2 and *Drosophila melanogaster* (dm) Ago1, a functional homologue of human Ago2, are different, probably due to differences in the intrinsic properties of human Ago2 and dmAgo1 or due to the different binding partners. Several lines of evidence support this idea. First, GW182 binding-domain of dmAgo1 and human Ago2 reside in PIWI domain [19,20]. The mutated regions of F2V2 reside in the Mid domain [10,11]. Therefore, it is possible that introduction of F2V2 into the Mid domain causes structural changes in dmAgo1 and thereby abolishes its interaction with GW182. On the other hand, it seems that the structure of hAgo2 is unaffected by these mutations and thereby Ago2F2V2 still associates with GW182 (Fig. 2B). Second, the cellular localizations of dmAgo1 and human Ago2 are different. DmAgo1 is localized in cytoplasm [21]. The ubiquitin associated-like domain (UBA) of GW182 is important for dmAgo1 localization to P-bodies [21], and overexpression of GW182 causes dmAgo1 to be localized in P-bodies [21]. In addition, several groups have shown that dmAgo1F2V2 mutant does not interact with GW182 and therefore it is not localized to P-bodies [11,21,22]. In contrast, human Ago2 is mainly localized to P-bodies [16,20,23–26], and the PAZ domain of hAgo2 is important for this localization [23]. It should be noted that mutations in the PAZ domain of dmAgo1 do not affect its P-body localization [22].

Recently, a merged model for mammalian NMD was proposed in which the termination codon is recognized as PTC by a competition between 3′UTR-associated factors, such as PABPC1, and EJC downstream of PTC [27–29]. EJC triggers the recruitment of NMD machinery to the terminating ribosome, whereas PABPC1 antagonizes the recruitment of NMD machinery, blocking the interaction between Upf1 and eRF3 [27–29]. In general, mRNAs harboring PTC generated by nonsense mutation have an extended 3′UTR compared to PTC-free mRNAs. According to the merged model, these mRNAs with long 3′UTRs would be subject to more efficient NMD. In addition, aberrant mRNAs with artificially extended 3′UTR are degraded by NMD [27,28,30–32]. However, numerous mRNAs with naturally occurring long 3′UTR escape NMD [27,32,33]. Although looping of the 3′UTR can shorten the spatial distance between the PABP-bound poly(A) tail and the terminating ribosome [28], mRNAs with long 3′UTR may contain a larger number of miRNA-binding sites at the 3′UTR. When Ago2/miRISC is loaded onto these miRNA-binding sites, these mRNAs would

escape NMD and instead would be silenced at the CT and ET steps by competition for cap-association between Ago2/miRISC and CBP80 and between Ago2/miRISC and eIF4E, respectively.

Acknowledgements

We thank Witold Filipowicz and Zissimos Mourelatos for providing Ago2-tethering plasmids and Marvin J. Fritzler for α-GW182 antibody. This work was supported by the Korea Science and Engineering Foundation (KOSEF) Grant funded by the Korea government (MEST) (2009-0084897, and 0001197) and the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2008-314-C00247).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.07.047.

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